Mode of action of famoxadone

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Abstract: Famoxadone is a preventative and curative fungicide recently developed for plant disease control. The molecule and its oxazolidinone analogs (OADs) are potent inhibitors of mitochondrial electron transport, specifically inhibiting the function of the enzyme ubiquinol:cytochrome c oxidoreductase (cytochrome bc_1). Visible absorbance spectral studies on the purified enzyme suggested that famoxadone bound close to the low potential heme of cytochrome b. This binding mode was confirmed in competitive binding experiments by studying the displacement of a radiolabelled OAD from submitochondria. EPR studies on the binding of famoxadone to submitochondria and purified bc_1 suggested its binding mode was more like that of myxothiazol than that of stigmatellin (ligands known to bind near the low potential heme). Zoospores of *Phytophthora infestans*, when given low concentrations of famoxadone and other OADs, were observed to cease oxygen consumption and motility within seconds and later the cells disintegrated, releasing the cellular contents. Famoxadone was a potent inhibitor of the growth of *Saccharomyces cerevisiae* when grown on non-fermentable carbon sources and it was an approximately 50-fold less potent inhibitor of growth when the yeast was grown on a fermentable carbon source, glucose. Such physiological observations are consistent with the loss of mitochondrial function imposed by famoxadone and OADs. Single amino acid changes in the apocytochrome b of baker's yeast cytochrome b located near the low potential heme altered the inhibition constants for the inhibitors famoxadone, myxothiazol, azoxystrobin and kresoxim-methyl differentially, thus strongly suggesting different binding interactions of the protein with the inhibitors.

Keywords: famoxadone; mode of action; fungicide; plant disease; cytochrome bc_1; enzyme inhibitors

1 INTRODUCTION

Famoxadone (3-anilino-5-methyl-5-(4-phenoxyphenyl)-3-1,3-oxazolidine-2,4-dione, Famoxate®, DPX-JE874) is a newly developed fungicide useful for preventative and curative control of fungal diseases in crops.[1] The fungicidal properties of this molecule were discovered through a chemical scouting and optimization methodology using enzyme and whole plant data rather than through an analog program against natural products or other known fungicides.[2] We report on studies which determined the mode of action of famoxadone and its oxazolidinone analogs (OADs) as a new chemical class of potent inhibitors of the catalytic function of mitochondrial cytochrome bc_1. Based on the history of the discovery of famoxadone and OADs there is little surprise that the molecules do not resemble other known inhibitors of mitochondrial cytochrome bc_1 (Fig 1).[3–10]

The function of mitochondrial bc_1 is inhibited by numerous synthetic and natural products,[3–5] inhibitors which have been classified into three groups according to their interactions with cytochrome bc_1. Group I inhibitors, myxothiazol, strobilurins, oudemansins and stigmatellins, interact with the low-potential heme of cytochrome b and are known as Q_o site ligands. The low-potential heme of cytochrome b is located near the outside of the mitochondrial inner membrane and the inhibitors displace ubiquinone from its role there. Stigmatellins differ from the other ligands in Group I in that they are thought to interact with the iron sulfur center of cytochrome bc_1 as well as with the low-potential heme. Group II inhibitors, the hydroxyquinones, interact with the iron sulfur center and not with cytochrome b, even though they are characterized as displacing inhibitors of Group I from their binding site. Group III inhibitors comprise the antimycins and funiculosins, which bind near the high-potential heme of cytochrome b and are known as Q_i site ligands because they displace ubiquinone from its location near the inside of the inner membrane. Cytochrome bc_1 is the

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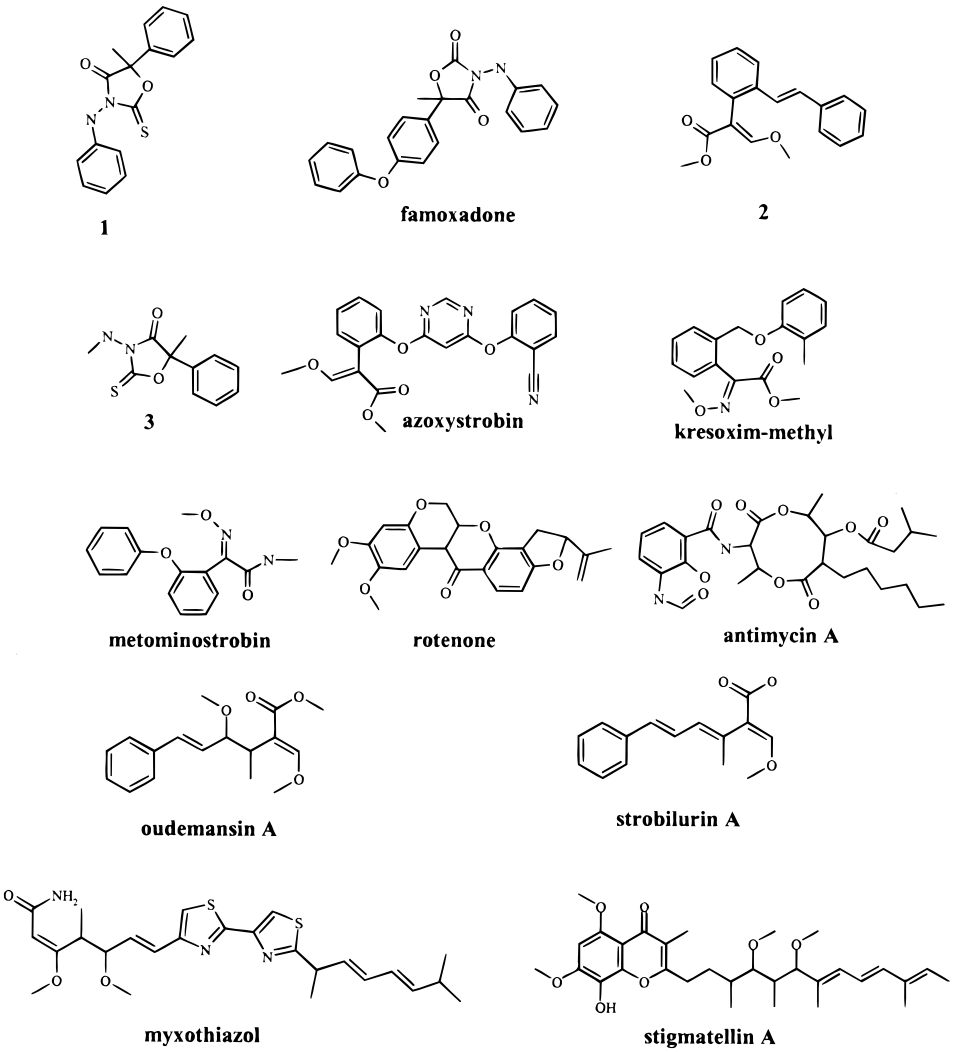

Figure 1. Inhibitors discussed in the text.

molecular target of two other newly developed fungicides used for plant disease control.[6–10] Azoxystrobin and kresoxim-methyl may be considered as analogs of the natural product strobilurin A, an inhibitor produced by the genus *Strobilurus* and some other fungi. These new crop-protection chemicals are improvements upon their natural product counterpart in terms of enhancing stability and other physical properties,[6–9] a worthy achievement by most measures.

The enzyme target (EC 1.10.2.2, ubiquinol:cytochrome c oxidoreductase, cytochrome bc_1, cytochrome c reductase, complex III, coupling site II) of mitochondrial electron transport (see Fig. 2) is the subject of numerous studies ranging from its recently solved atomic structure[11] to its disfunction as a cause of human diseases.[12] The enzyme is also central to the classical treatise of Mitchell on the proton motive force and the energy conservation cycle.[13] The enzyme functions as a dimer of 2 × 12 individual subunits which has been the subject of biochemical studies[14] based on Mitchell's theory and which has recently been corroborated by the 3D structure reported by Xia *et al.*[11]

2 MATERIALS AND METHODS

2.1 Materials

Yeast isolates having single amino acid changes in apocytochrome b were a kind gift from Dr A-M Colson and have been described in detail elsewhere.[15] Antimycin A, rotenone, myxothiazol and stigmatellin were from Sigma (St. Louis, MO). [^{14}C] Phenylalanine, [^{14}C] uridine and [^{3}H]thymidine were from DuPont NEN (Cambridge, MA). Preparation of other inhibitors was as described[2] or will be described elsewhere. Other materials, unless specified, were from Aldrich Chemical (Milwaukee, WI), Sigma Chemical (St. Louis, MO), or VWR (Trenton, NJ). Non-linear least-squares analyses were through the computer program RS1 (BBN Research Systems; Cambridge, MA). Glass beads, bead beaters and mini bead beaters were from Biospec Products (Bartlesville, OK). Radioactivity in samples was determined by scintillation counting in a Beckman LS3801 instrument (Beckman, Fullerton, CA) with an external standard method for correcting for counting efficiency.

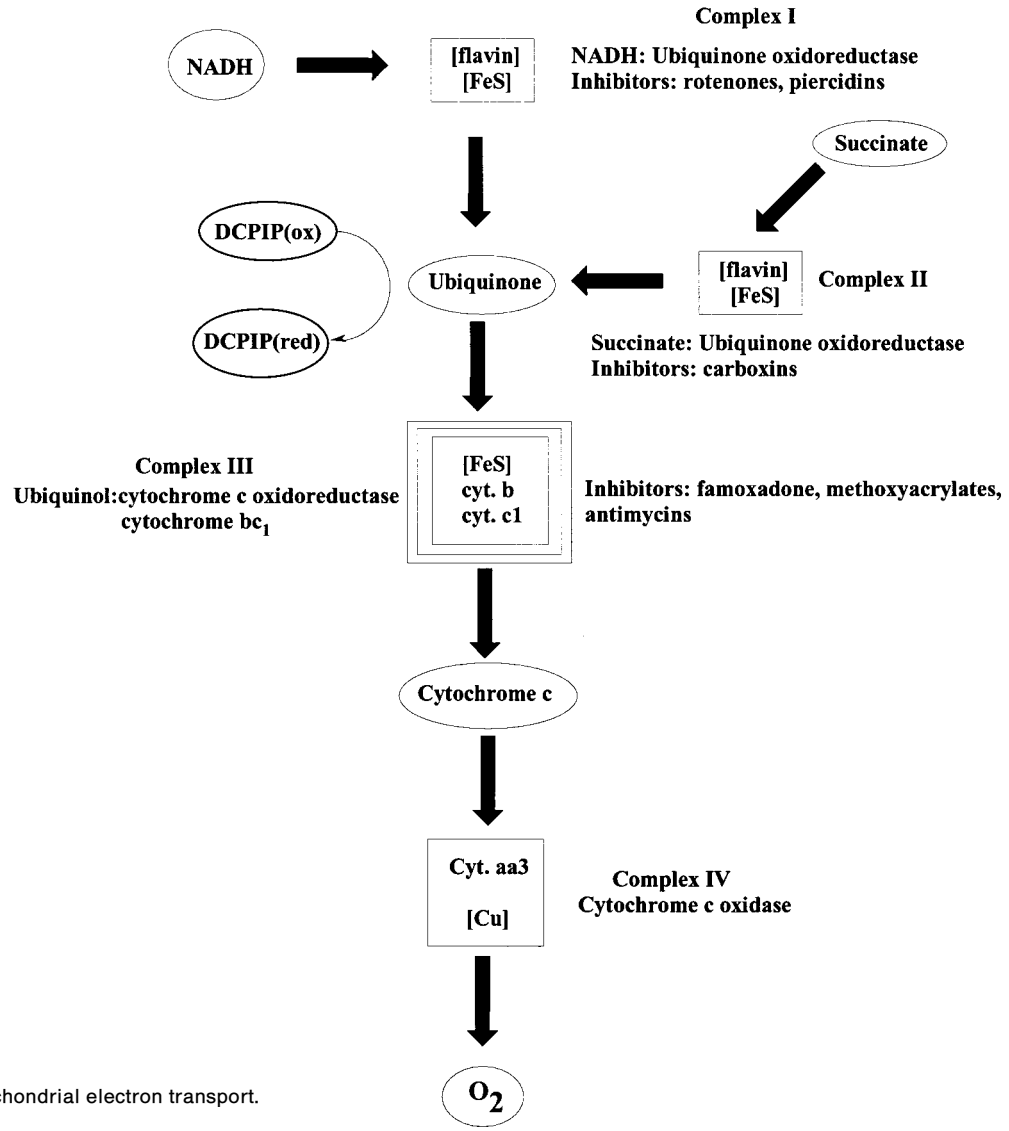

Figure 2. Mitochondrial electron transport.

2.2 Methods

2.2.1 Mitochondrial preparations

All buffers and purification procedures described in this section were at 0–4°C.

2.2.1.1 Rat heart mitochondria. Rat hearts were isolated by using an established procedure at Haskell Laboratory (Newark, DE). Immediately after extraction the hearts were placed in sodium chloride solution (20 g litre^{-1}) and then washed with Buffer A, containing sucrose (250 mM) Hepes-NaOH (20 mM) and EDTA (0.1 mM; pH 7.5). The hearts were minced with a razor blade and homogenized in Buffer A (1 + 4, w/v) using a Dounce homogenizer with a loosely-fitting pestle followed by a ground glass pestle. The homogenate was centrifuged for 10 min at 900*g*. The supernatant was collected and centrifuged for 40 min at 30 000*g*. The resulting submitochondrial pellet was homogenized in Buffer A to 20 mg protein ml^{-1}, flash frozen in liquid nitrogen, and stored at −80°C until use.

2.2.1.2 Beef heart mitochondria. Beef hearts were obtained from a local slaughterhouse where, after isolation, they were chilled in sodium phosphate (50 mM; pH 7.0) + NaCl (0.1 M). The meat was minced followed by homogenization in Buffer A (1 + 4, w/v) using a Waring Blender. The homogenate was centrifuged for 10 min at 1000*g*. The resulting supernatant was adjusted to pH 7.5 with Tris base (2.0 M) followed by centrifugation for 30 min at 15 000*g*. The submitochondrial pellet was homogenized in Buffer A to 40 mg protein ml^{-1}, flash frozen in liquid nitrogen, and stored at −80°C until use.

2.2.1.3 Yeast mitochondria. One liter of yeast cells (strains listed in Section 1) were grown overnight in a 2-liter flask containing yeast peptone glycerol (YPG) medium: yeast extract (10 g liter^{-1}), Bactopeptone (10 g liter^{-1}) and glycerol (20 g liter^{-1}), at 30°C with shaking at 200 rev min^{-1}. Cultures were then centrifuged at 1000*g* for 20 min. Pelleted cells were subsequently washed in cold water followed by recentrifugation as above. Washed cells were suspended 1 + 2 (w/v) in cold lysis buffer Tris-HCl (10 mM; pH 7.4), sorbitol (0.6 M) and EDTA (1 mM). Cells were lysed using a mini-bead beater with mini-bead beater tubes filled halfway with 0.5-mm glass beads, with three 1-min bursts and cooling on wet ice between bursts. Complete lysis was confirmed by

microscopic observation. The cell lysate was centrifuged at 400*g* for 10 min to pellet unbroken cells, nuclei and cell debris. The supernatant was centrifuged at 16 000*g* for 20 min to pellet mitochondria. The mitochondrial pellet was resuspended in cold lysis buffer plus BSA (1.0 g liter^{-1}), flash frozen in liquid nitrogen, and stored at −80°C until use.

2.2.1.4 Magnaporthe grisea mitochondria. Cultures of *Magnaporthe grisea* (Hebert) ME Barr were grown for 48 h in liquid medium[16] at 30°C with shaking at 200 rev min^{-1}. Mycelial mass was collected by vacuum filtration and washed with water at 0°C. Cells were disrupted in 4 volumes of Buffer A by using a bead beater with five 45-s bursts with 2-min cooling periods on wet ice between bursts. Lysed cells were centrifuged for 15 min at 500*g*. The supernatant was centrifuged for 20 min at 27 000*g* and the resulting mitochondrial pellet was suspended in Buffer A plus BSA (1 mg ml^{-1}). Aliquots were flash frozen in liquid nitrogen and stored at −80°C until use.

2.2.1.5 Achlya sp. mitochondria. Zoospores of *Achlya sp.* strain #52867 (obtained from Dr James Steffens, DuPont Agricultural Products) were used to inoculate 1 liter of medium containing yeast peptone dextrose (YPD) at pH 6.5. The culture was grown for 40 h at 30°C with shaking at 200 rev min^{-1}. Fungal material was collected by filtration followed by disruption in Buffer A by using a bead beater. The lysate was centrifuged for 15 min at 2500*g*. The supernatant was centrifuged for 20 min at 15 000*g* to pellet mitochondria. The resulting pellet was homogenized in a minimal volume of buffer [Tris-HCl (10 mM; pH 7.4), sucrose 0.8 M, Na_2EDTA (1 mM) and BSA (1 g liter^{-1})], frozen in liquid nitrogen and stored at −80°C until use.

2.2.1.6 Cauliflower mitochondria. Cauliflower mitochondria were prepared by using a Percoll gradient as described elsewhere.[17] Mitochondrial fractions from the Percoll gradient were mixed 1 + 15 (w/v) with mannitol (0.3 M), MOPS-NaOH (10 mM; pH 7.4) EDTA (1 mM) and BSA (1 g liter^{-1}) and centrifuged for 30 min at 40 000*g*. The mitochondrial pellet was suspended in the same buffer, frozen in liquid nitrogen and stored at −80°C until use.

2.2.1.7 Neurospora crassa mitochondria. Cultures of *Neurospora crassa* Shear & Dodge were initiated by adding 10^9 spores to 1 liter of Vogel's medium[18] containing sucrose (20 g liter^{-1}). Cultures were grown for 16 h at 30°C with shaking at 250 rev min^{-1}, then placed in a wet ice bath to chill, followed by vacuum filtration to collect mycelial mass. Mycelium was washed with water. The fungal material was disrupted in Hepes-NaOH (0.1 M; pH 7.5), glycerol (200 g liter^{-1}), EDTA (1 mM), DTT (1 mM); (1 + 5 w/v); using a 350-ml bead beater with five 45-s bursts with 2-min cooling periods on wet ice between bursts. Lysate was centrifuged for 15 min at 450*g*. The supernatant was centrifuged for 20 min at 15 000*g* and the resulting mitochondrial pellet was suspended in the disruption buffer, frozen in liquid nitrogen and stored at −80°C until use.

2.2.1.8 Phytophthora infestans mitochondria. Phytophthora infestans (Mont.) de Bary sporangia were isolated by washing V8 agar[19] plates with cold water and filtering the washings through glass wool. Sporangia were incubated at 12°C for 1–4 h and were observed under a microscope to determine when zoospores were being released. When many zoospores were observed aliquots of culture medium were mixed 1 + 1 by volume with Buffer A. Following two 30-s bursts in a mini bead beater, the lysate was centrifuged for 10 min at 600*g*. The supernatant was centrifuged for 20 min at 20 000*g* and the mitochondrial pellet was suspended in a minimal volume of Buffer A. Mitochondria were used for inhibition studies within 1–2 h of isolation.

2.2.1.9 Beef heart cytochrome bc_1. Active beef heart bc_1 complex was purified in buffer containing Triton X-100 by the method of Schägger *et al.*[20] For Preparation A the final purification step was gel filtration through a Sepharose Cl-6B column equilibrated with Mops-NaOH (20 mM), NaCl (100 mM), Triton X-100 (0.5 g liter^{-1}) and NaN_3 (2.0 mM). The enzyme was flash frozen in column buffer and stored at −80°C until use. Analysis of the purified complex by SDS-PAGE[21] indicated a pure enzyme. Preparation B was by the same method except that the Sepharose Cl-6B column step was omitted. The enzyme in hydroxyapatite buffer [potassium phosphate (200 mM; pH 7.2), Triton X-100 (2.5 g liter^{-1}), NaN_3 (2.0 mM)] was frozen in liquid nitrogen and stored at −80°C until use.

Fe-S deficient cytochrome bc_1 was prepared in the presence of antimycin A as described elsewhere.[20] The purified complex in hydroxyapatite buffer [sodium phosphate (200 mM; pH 7.2), NaN_3 (2 mM), Triton X-100 (0.5 g liter^{-1})] was frozen in liquid nitrogen and stored at −80°C until use.

2.2.2 Measurement of red shift difference absorbance spectra of bc_1-inhibitor complexes

Red shift spectra were recorded on an HP 8542A diode array spectrophotometer (Hewlett Packard) as the dithionite-reduced enzyme in the absence and presence of inhibitors. Cytochrome b contents of the cytochrome bc_1 complexes studied were determined from the difference spectra of reduced-oxidized enzyme at 562–574 nm ($\varepsilon = 28.5$ mM^{-1} cm^{-1}). Cytochrome bc_1 (0.7 ml in a quartz cuvette) in its column buffer as stored was scanned by the spectrophotometer. Enzyme was fully reduced with two grains of sodium dithionite and was scanned by the spectrophotometer. Inhibitor (in 2 µl ethanol) was added to the reduced enzyme and was scanned by the spectrophotometer. Further additions of inhibitor(s) in 2 µl ethanol were made and the enzyme was scanned by the spectrophotometer at each stage. All wavelength scans by the spectrophotometer were stored digitally and difference spectra were produced by subtracting

spectra obtained from a single cuvette incubation from one another.

2.2.3 Determination of enzyme inhibition constants
All enzyme reactions described below were at 25°C and rates were monitored by using an HP 8542A diode array spectrophotometer. Linear initial rates were fitted to a line using the spectrophotometer's software. Inhibition constants were determined by fitting rates to eqn (1), where v is the observed initial rate, V is the uninhibited rate, I is the inhibitor concentration and IC_{50} is the concentration of inhibitor which gives 50% inhibition.

$$v = \frac{V}{1 + I/IC_{50}} \quad (1)$$

2.2.3.1 NADH to O_2. Reaction mixtures (1 ml) were held in quartz cuvettes and contained potassium phosphate (0.1 M) and cytochrome c (10 μM) at pH 7.5. Submitochondria (5–20 μg protein) was added to the reaction mixture 10 min before the addition of inhibitor (in 5 μl ethanol) which was immediately followed by the addition of 100 nmol NADH in 10 μl. The reaction (NADH to O_2) was monitored at 340 nm continuously for 1 min.
2.2.3.2 NADH to 2,6-dichlorophenol-indophenol (DCPIP) (complex I). Reaction mixtures (1 ml) contained potassium phosphate (100 mM; pH 7.5), NADH (0.1 mM), DCPIP (0.075 mM), inhibitor in ethanol (5 μl), and beef heart submitochondria (20 μg). Reactions were initiated with NADH (0.1 mM; 10 μl) and reduction to DCPIP was followed for 1 min continuously at 600 nm. Under these conditions rotenone had an IC_{50} of 3.9 (±0.05) ng ml^{-1}.
2.2.3.3 Succinate to DCPIP (complex II). Reaction mixtures (1 ml) contained potassium phosphate (100 mM; pH 7.5), potassium succinate (20 mM) DCPIP (0.075 mM), inhibitor in ethanol (5 μl), and beef heart submitochondria (40 μg). Reactions were initiated with succinate (20 mM; 40 μl) and reduction of DCPIP was monitored for 1 min continuously at 600 nm.
2.2.3.4 Succinate to cytochrome c (complexes II and III). Reaction mixtures (1 ml) contained potassium phosphate (100 mM; pH 7.5), succinate (20 mM), cytochrome c (40 μM), KCN (5 mM), inhibitor in ethanol (5 μl), and beef heart submitochondria (10 μg). Reactions were initiated with succinate (20 mM; 40 μl) and reduction of cytochrome c was followed continuously for 1 min at 550 nm.
2.2.3.5 Cytochrome c to O_2 (complex IV). Reaction mixtures (1 ml) contained potassium phosphate (100 mM; pH 7.5), reduced cytochrome c (40 μM), inhibitor in ethanol (5 μl), and beef heart submitochondria (10 μg). Reactions were initiated with cytochrome c (40 μM; 50 μl) and oxidation of cytochrome c was followed for 1 min continuously at 550 nm.
2.2.3.6. Ubiquinol to cytochrome c (complex III). Reaction mixtures (1 ml) contained potassium phosphate (0.1 mM), KCN (1.0 mM) and, cytochrome c (42 μM). An aliquot (5 μl) of ethanol with and without inhibitor was added to the mixture followed by an aliquot (5 μl) which included 2.3 pmol of purified beef heart cytochrome bc_1 in Mops-KOH (20 mM; pH 7.2), KCl (0.1 mM), NaN_3 (2 mM), Tween-20 (2.0 g $liter^{-1}$), Triton X-100 (0.3 g $liter^{-1}$), BSA (5 g $liter^{-1}$) and glycerol (50 g $liter^{-1}$). Reactions were initiated by adding 5 μl which held 20 nmol reduced coenzyme Q-2[22] in water and the reaction was monitored continuously for 10 s at 550 nm.

*2.2.4 Preparation of [^3H] **1***
The synthesis is described in Fig 3. [^{3}H] Ethyl-atrolactate was prepared at NEN as follows. Ethyl-4-bromoatrolactate, (26 mg), was dissolved in a solution of sodium acetate trihydrate (100 mg) in ethanol (5 ml). To this was added 50 mg of 10% Pd/C and one atmosphere of tritium gas. The reaction was stirred at room temperature for 1 h and an uptake of 4 ml of tritium gas was observed. Labiles were removed with ethanol. Approximately 20 mCi of the carrier-free labeled atrolactate was provided to us as a 30-ml solution in tetrahydrofuran (THF). Analysis of this sample by HPLC (ODS Reliance-3, methanol + 1 g $liter^{-1}$ H_3PO_4 in water (50 + 50 by volume) 2 ml min^{-1}, 40°C) with UV/radioactive detectors showed a single radioactive peak with a retention time (1.53 min) corresponding to ethyl atrolactate.

A 5.0-ml sample of the tritiated ethyl atrolactate solution was concentrated under a stream of nitrogen. The residue was diluted with 5.0 ml of *n*-butyl chloride and 50 μl (5.0 mg, 25.7 μmol) of carrier ethyl atrolactate in THF was added. A 10-μl aliquot of a 1 : 100 dilution of this solution gave 184,230 dpm (total counts: 9.22×10^9 dpm, 4.15 mCi, specific activity = 4.15 mCi/25.7 μmol = 160 Ci mol^{-1}). The *n*-butyl chloride solution was then washed with 1 ml each of hydrochloric acid (1 M), saturated sodium bicarbonate solution and brine, dried over $MgSO_4$, filtered and concentrated under a stream of nitrogen. The residual oil was taken up in 100 μl of THF and added to 5.1 mg of potassium *tert*-butoxide in a 1-ml conical vial. After stirring at room temperature for 10 min, 40 μl of carbon disulfide solution

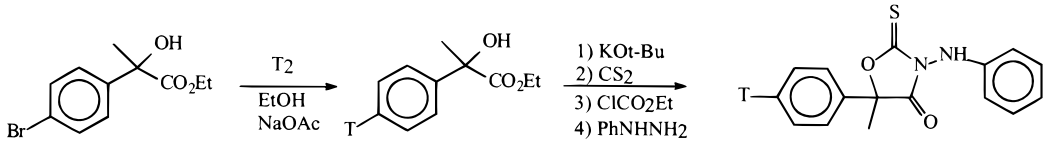

Figure 3. Scheme for preparation of [^{3}H]**1**.

(76 mg ml^{-1} in THF) was added and stirred for 5 min; 40 µl of ethyl chloroformate solution (109 mg ml^{-1} in THF) was added, stirred for 5 min followed by the addition of 40 µl of phenyl hydrazine solution (108 mg ml^{-1} in THF). The resulting reaction mixture was stirred at room temperature for 1 h, diluted with 8.0 ml of *n*-butyl chloride, washed with 2 ml of hydrochloric acid (1 M) and dried ($MgSO_4$). Analysis of the crude product by HPLC showed that the labeled **1** accounted for 12.7% of the total radioactivity of the sample. Purification by preparative silica gel TLC yielded a total of 413×10^6 dpm of **1** (4.5% yield, 345 µg). HPLC (employing a radioactive flow-through detector) showed the product to be 85% pure, the remainder being a single, more hydrophobic impurity.

Final purification of [^{3}H] **1** was achieved by using HPLC and a reverse phase column (ODS) with a gradient of acetonitrile to methanol. The tritiated material was >95% pure from comparisons of expected (based on dpm) and found results using UV spectroscopy and IC_{50} determinations (data not shown).

*2.2.5. Displacement of [^3H] **1** in competitive binding studies*

Reaction mixtures (1 ml) contained rat heart submitochondria (0.5 mg protein), [^{3}H] **1** (10 000 dpm; approximately 28 nM), and other additions as indicated, in sucrose (200 mM) and Tris-HCl (100 mM; pH 7.5). After incubating the mixtures for 10 min at 25°C, they were centrifuged 10 min at 10 000*g* and 4°C in a swinging bucket rotor to fully pellet the submitochondria. An aliquot of the supernatant was removed and added to 10 ml of Aquasol® scintillation cocktail for determination of radioactivity. Radioactivity in the supernatant of the positive control which included 34 µM unlabelled **1** in the 10 min incubation was compared to the radioactivity in the negative control, which had no further additions, to give a value for the specifically bound dpm (specifically bound dpm = positive control dpm − negative control dpm). Specifically bound dpm values ranged from 500 to 700, equal to 20–25% of the negative control dpm values in the six independent experiments conducted by this protocol. For comparison of the effects of added ligands to the incubations, the data were expressed as a fraction of the specifically bound dpm retained, which equalled specifically bound dpm in the presence of the ligand (such as myxothiazol, antimycin A and others) divided by the specifically bound dpm in the absence of added ligand. Binding experiments were conducted in triplicate within the independent trials, except where noted.

2.2.6 EPR spectra of inhibitor complexes

EPR spectra were recorded at 15 K by using an Oxford ESR-901 helium-flow cryostat on a Bruker ER200D spectrometer equipped with an upgraded computer software system (ESP-300 Model).The bc_1 complex isolated from beef heart mitochondria with Triton X-100 was dissolved in Mops-NaOH (30 mM; pH 7.2) buffer containing NaCl (100 mM) and Triton X-100 (1.0 g liter^{-1}) at a final concentration of 0.025 mM cytochrome c_1. The complex was reduced by adding 5 mM ascorbate, and treated with 0.1 mM of various inhibitors dissolved in ethanol. The samples were incubated at 0°C for 10 min before being placed in EPR tubes and frozen in liquid nitrogen.

2.2.7 Inhibition of growth in fungi

2.2.7.1 Yeast growth assays on varying carbon sources. Three 50-ml cultures of *Saccharomyces cerevisiae* Meyer ex Hansen, (isolate S228C) were growth overnight at 30°C in YPD (glucose), YPE (ethanol), or YPG (glycerol) media which contained yeast extract (10 g liter^{-1}), Bactopeptone (10 g liter^{-1}) and the indicated carbon source (20 g liter^{-1}). Cells were harvested by centrifuging for 10 min at 800*g* in a Sorvall RT6000 centrifuge. Pelleted cells were resuspended in fresh and respective growth media and centrifuged as above. The washed cell pellets were suspended in respective media to an OD_{600} of 0.01. Inhibition-of-growth assays were conducted in 96-well microtiter plates (Falcon #3072). Aliquots (0.15 ml) of diluted cell suspensions were placed in wells of row A and 0.1-ml aliquots were placed in wells of the remaining rows. Inhibitors (5 µl, solubilized at 0.3 mg ml^{-1} in ethanol) were added to wells of row A by using a 10-µl Hamilton syringe. The cells and inhibitors in row A were mixed by using a multichannel pipetor. Serial dilutions of the inhibitors were achieved by pipeting and mixing 50 µl from row A into row B followed by doing the same for rows B and C and C and D and so on until the final concentrations of the inhibitors on the plate ranged from 10 000 ng ml^{-1} to 4.6 ng ml^{-1} from rows A to H. The microtiter plates were incubated 20 h at 30°C, after which OD_{450} values were read by using a plate reader (Molecular Devices, Inc). Growth data were fitted to eqn (1) where, in this case, *v* was the measured OD_{450} values of the wells containing inhibitor and *V* was the OD_{450} value of the wells lacking inhibitor.

2.2.7.2 Growth studies on filamentous fungi. All fungi listed in Table 8 were grown in liquid culture in 96-well microtiter plates at 24°C and constant humidity. *P. infestans* was grown in Henninger's medium.[23] All other fungi listed in Table 8 were grown in a medium of the following composition: salts: K_2HPO_4 (3), KH_2PO_4 (4), NaCl (0.5), NH_4Cl (1), $MgSO_4 \cdot 7H_2O$ (0.2), $CaCl_2 \cdot 2H_2O$ (0.01 g liter^{-1}). Trace elements: $MnSO_4 \cdot H_2O$ (0.1), $ZnSO_4 \cdot 7H_2O$ (0.2), $CuSO_4 \cdot 5H_2O$ (0.2), $FeSO_4 \cdot 7H_2O$ (0.2), $Na_2MoO_4 \cdot 2H_2O$ (0.1), $CoSO_4$ (0.06) and H_3BO_3 (0.08 mg liter^{-1}); vitamins; biotin, (0.005 mg liter^{-1}); glucose (10 g liter^{-1}). Famoxazone and azoxystrobin were dissolved in dimethyl

sulfoxide (DMSO) as stock solutions (1.2 mg ml^{-1}). An aliquot (2.5 µl) of the stocks or DMSO controls was added to each well (150 µl medium per well) in the first row of a microtiter plate. The remainder of the plate contained 100 µl medium per well. Beginning with the first row, standards were serially diluted row-wise down the plate resulting in eight dilutions (1 : 2, 50 µl added to 100 µl each transfer). The last 50 µl from the final row was discarded. Propagules were obtained from filamentous fungi (except *Rhizoctonia solani*) by careful washing of plates or flasks with medium containing Tween 20 (0.1 g liter^{-1}). Both *N. crassa* and *P. infestans* were isolated as above but without Tween in the medium. Plate washes were then filtered through glass wool and propagules counted using a hemacytometer and diluted appropriately. Inoculum was prepared from *R. solani* by blending an entire agar mat with 50 ml of medium containing Tween-20 (0.1 g liter^{-1}) for 4 × 5-s pulses (low speed) in a Waring blender. The suspension was centrifuged for 1 min at 2000 rev min^{-1} (Beckman RT-2000 tabletop centrifuge). The supernatant was removed from the viscous agar pellet and diluted 1 to 10 with fresh medium and used as the inoculum. One hundred microliters of inoculum or propagules for all fungi were then added to the 96-well test plate, resulting in a 200-µl final assay volume. Inoculum concentration for all fungi was approximately 10^4 propagules ml^{-1} except for *R. solani* which was a 1 to 10 dilution of the stock preparation described above. The culturing periods in days at the time of IC_{50} determination were 2 for *R. solani*, 4 for *M. grisea*, 3 for *S. nodorum*, 6 for *P. infestans* and 2 for *N. crassa*. Growth inhibition was assessed by monitoring differences in optical density (OD) at 450 nm relative to controls. OD was measured on a microtiter plate reader (Molecular Devices, Inc) and IC_{50} values calculated from the digitized data according to eqn (1).

2.2.8 *Incorporation of radiolabelled precursors into macromolecules*

Cultures of *P. infestans* were grown in V8 complex medium[19] at 20°C. The cultures were diluted into fresh medium to 0.1 g fresh weight mycelial mass to 10 ml medium. Aliquots (0.1 ml) of ethanol solutions containing 1 mg ml^{-1} **1**, metalaxyl, or without compound were added to the 10-ml cultures. Incubation of the cultures continued for 0.75 h at 20°C with shaking at 200 rev min^{-1} before addition of 1.0 µCi of radiolabelled precursors uridine, thymidine, or phenylalanine. After 0.5-h incubations with radiolabels at 20°C with shaking, incorporation was stopped by adding trichloroacetic acid + water (50 + 50 by volume; 5 ml) at 0°C to each 10-ml culture. Cultures were placed on ice for 1 h and were then harvested by filtration on GFA glass filters under vacuum followed by washing with trichloroacetic acid + water (10 + 90 by volume; 30 ml) followed by ethanol + ethyl ether (1 + 1 by volume; 20 ml), all at 0°C. The resulting mass on the filter was analyzed using scintillation counting methods. Results are presented as a percentage of uninhibited controls. Dpm values for incorporation into uninhibited controls were: [^{14}C]uridine, 96 000 (mean of three independent experiments having duplicate incubations); [^{3}H]thymidine, 3600 (average of duplicate incubations and [^{14}C] phenylalanine, 1 100 000 (average from duplicate incubations).

2.2.9 *Inhibition of oxygen uptake by* Phytophthora infestans *zoospores*

Zoospores were raised as described in Section 2.2.1.8. Aliquots (1.0 ml) of the zoospore suspension in water were added to a Hansatech oxygen electrode chamber equilibrated at 15°C. Rates of oxygen consumption were measured in the absence of inhibitor for several minutes followed by the addition of inhibitor (in 1.0 µl ethanol) and oxygen consumption was measured for an additional several minutes. IC_{50} values were fitted to eqn (1) where v was the measured rate of oxygen consumption in the presence of inhibitor, V the rate of oxygen consumption in the absence of inhibitor, and the other definitions remained as in Section 2.2.3.

2.2.10 *Zoospore disruption studies*

2.2.10.1 Plasmopara viticola *zoospores. P. viticola* was induced to sporulate by placing diseased grape plants of (cv Chardonnay) in a dew chamber at 20°C for 24 h. After 24 h, copious sporangia were visible on abaxial leaf surfaces. Sporangia were collected by rinsing several leaves with water and filtering the suspension through a small plug of glass wool. Sporangia were incubated at 20°C for 30 min, at which time they began to differentiate and release zoospores. A 5 µl drop of this sporangia/zoospore suspension was placed in the center of a glass microscope slide. A 5 µl drop of an aqueous solution containing famoxadone at 4 µg ml^{-1} was added directly to the suspension, giving a final concentration of 2 µg famoxadone ml^{-1}. A cover glass was applied to the drop and the edges sealed with paraffin. The suspension was viewed using an Olympus BX-60 microscope equipped with Nomarski differential interference contrast optics and a 100× oil immersion objective lens. Photographs were taken with an Olympus OM-4Ti camera using Kodak Ekta Elite film.

2.2.10.2 Phytophthora infestans *zoospores. P. infestans* 580z.1 isolate (provided by W Fry, Cornell University; z.1 represents single zoospore isolation) was grown on V8 agar[19] for seven days at 20°C. The fungus grew as aerial hyphae and produced copious sporangia under these conditions. After seven days, sporangia were collected by flooding the plate with 20 ml of 4°C sterile water and dislodging sporangia with a sterile camel hair brush. The sporangial suspension was poured through a sterile funnel fitted with a small plug of sterile glass wool to remove

mycelial fragments. The resulting sporangial suspension was transferred to the wells of a 96-well microtiter plates for inhibitor treatments. Serial dilutions of inhibitors were placed at 2 × final concentration in 100 µl of sterile water in wells of a sterile 96-well microtiter plates. The sporangial suspension was added to all wells of the plate, resulting in a final assay volume of 200 µl per well, and testing a range of inhibitor concentrations from 10 µg ml^{-1} to 0.01 µg ml^{-1} in 1 : 1 dilutions per step. Assay plates were incubated at 15°C for up to 4 h. Microscopic assessment of sporangial differentiation and zoospore release began at 2 h after incubation and was done on a Leitz Fluovert inverted microscope using Hoffman Modulation Contrast optics with a 40 × objective lens and fitted with an Olympus OM4-Ti camera using Kodak Ekta Elite color slide film.

2.2.11 Disease control evaluations

Aqueous suspensions of test compounds were sprayed to the point of run-off onto grape seedlings and afterwards the plants were placed in a growth chamber. The following day the seedlings were inoculated with a spore suspension of *Plasmopara viticola* Berl. & de Toni, (the causal agent of grape downy mildew) and then incubated in a water-saturated atmosphere for 24 h at 20°C. The plants were transferred to another growth chamber for six days of incubation at 20°C, followed by incubation for 24 h in a water-saturated atmosphere at 20°C, after which disease ratings were made.

3 RESULTS

3.1 Early mode of action studies on fungicide 1

The initial chemical lead in the discovery of famoxadone was molecule **1** which showed excellent fungicidal properties in greenhouse tests in 1988. Early biochemical studies on **1** suggested it was a general poison to central metabolism in fungi. Incubations of mycelium of *P. infestans* with **1** for 0.75 h strongly inhibited the incorporation of radiolabelled precursors amino acids, ribonucleotides and deoxyribonucleotides into their respective polymers, protein, RNA and DNA (Table 1). Metalaxyl was included as a positive control for inhibition of radiolabelled uridine incorporation because it has been reported to be an inhibitor of RNA synthesis in *Pythium splendens* Braun[24] and specifically to inhibit a RNA polymerase in *Phytophthora megasperma* Drechs.[25] We observed very little inhibition of RNA synthesis in *P. infestans* treated with metalaxyl, possibly because the strain we employed in the studies was resistant to the fungicide. Incubations of *Ustilago maydis* (DC) Corda cells for 1 h with **1** prevented the incorporation of radiolabelled acetate into the sterol biosynthetic pathway (measuring intermediates from squalene to ergosterol) (data not shown).

Next, fungicide **1** was examined against a number of fungicide enzyme targets under study, including

Table 1. Inhibition of the incorporation of radiolabelled precursors into macromolecules in *Phytophthora infestans* by **1** and metalaxyl[a]

Molecule	*Uridine*	*Thymidine*	*Phenylalanine*
1	0.14 (±0.02)[b]	0.20[c]	0.25[c]
Metalaxyl	0.84 (±0.11)[b]	ND[d]	ND[d]

[a] Values are expressed as a fraction of the control incubations which lacked inhibitor.
[b] Means and standard deviations from three independent studies having two incubations for each treatment.
[c] Averaged values of two incubations from a single study.
[d] ND, not determined.

cytochrome P450 reductase, cytochrome b_5 reductase, squalene epoxidase, isoprenyl pyrophosphate isomerase, dihydroorotase and dihydroorotate dehydrogenase, where there was no inhibition of the measured activities at 5–10 µg ml^{-1} **1**. In turn, **1** was found to be a potent inhibitor of mitochondrial electron transport (NADH to O_2) in rat heart submitochondria (Table 2). Mitochondrial electron transport inhibitor **1** was found to be a weak inhibitor of complex I, complex II and complex IV in rat heart mitochondria and a strong inhibitor of reactions through complex I and III and complexes II and III, thus implicating complex III as the site of inhibition (Table 1, see Fig. 2). Spectral studies on beef heart submitochondria incubated with **1** and NADH as an electron donor indicated an increased absorbance at about 565 nm in comparison to the untreated control, further substantiating the site of inhibition by **1** as being at cytochrome b of the bc_1 complex (complex III). However, the spectral definition was insufficient for differentiating the binding site of **1** between the Q_o or Q_i sites of cytochrome bc_1.

Chiral synthesis of the enantiomers of **1** allowed an independent assessment of the in-vitro assignment of

Table 2. Inhibition of individual segments of the mitochondrial electron transport chain by **1** and famoxadone in beef heart mitochondria

	IC_{50} (ng ml^{-1}) (±SEM)	
Assay	***1***	*Famoxadone*
NADH to O_2	49 (±1)	8.8 (±0.5)
NADH to cytochrome c	440 (±30)	41 (±2)
Succinate to cytochrome c	510 (±8)	40 (±1)
Cytochrome c to O_2	NI[a]	NI[a]
NADH to DCPIP	4700 (±80)	3600 (±60)
Succinate to DCPIP	19 000 (±4000)	NI[a]
Ubiquinol to cytochrome c[b]	ND[c]	28 (±0.6)

[a] NI, no inhibition at 10 000 ng ml^{-1}.
[b] Results from purified beef heart cytochrome bc_1.
[c] ND, not determined.

Table 3. Selectivity of enantiomers of **1** for inhibition of mitochondrial electron transport (NADH to O_2) in submitochondria isolated from rat heart and *Phytophthora infestans*, inhibition of growth in *Phytophthora infestans*, and control of grape downy mildew[a]

Compound	*Electron transport IC_{50} (ng ml^{-1})*		*Growth IC_{50} (ng ml^{-1})*	*Disease control ED_{90} (μg ml^{-1})*[b]
	Rat heart	*P. infestans*		
1 (*R*, *S*)	45 (±3)[a]	13 (±2)	50	10
1 (*R*+)	1400 (±20)	450 (±14)	9000	80
1 (*S*−)	17 (±2)	3.8 (±0.9)	15	3.0

[a] Mean and standard deviation from five determinations.
[b] ED_{90} values were estimated from plant disease control reports (DuPont Agricultural Products).

inhibition of cytochrome bc_1 against its relevance to its fungicidal effects. The *R*+ enantiomer was a 1000-fold weaker inhibitor of mitochondrial electron transport than the active *S*− enantiomer (Table 3). (*S*−) **1** was approximately 1000-fold more potent as an inhibitor of growth in *P. infestans* than its *R*+ counterpart. Also, greenhouse results indicated (*S*−) **1** was much more potent than (*R*+) **1** in protecting plants from grape downy mildew.

Purified beef heart cytochrome bc_1 reduced with dithionite produced the classical red shift of known inhibitors[5,26] upon treatment with **1** as indicated from the difference spectrum (Fig 4). Difference spectra of the reduced cytochrome bc_1 more resembled that produced by myxothiazol than that produced by antimycin A: the red shift trough and peaks with both **1** and myxothiazol were consistently at 560 and 566 nm, respectively; the red shift trough and peak with antimycin A were consistently at 558 and 564 nm, respectively. Furthermore, addition of myxothiazol to the reduced cytochrome bc_1 saturated with 10 μg ml^{-1} **1** produced no larger difference spectrum in the 500–600 nm region than before the addition. However, addition of antimycin A to the reduced cytochrome bc_1 saturated with 10 μg ml^{-1} **1** increased the difference spectrum to the extent that the spectrum of the reduced cytochrome bc_1 saturated with 10 μg ml^{-1} **1** and the reduced cytochrome bc_1 saturated with 10 μg ml^{-1} antimycin A alone were nearly additive in the spectrum of the reduced cytochrome bc_1 saturated with both inhibitors simultaneously. These spectral results suggested that **1** occupied an overlapping binding site with myxothiazol and **1** occupied an entirely independent binding site from antimycin A.

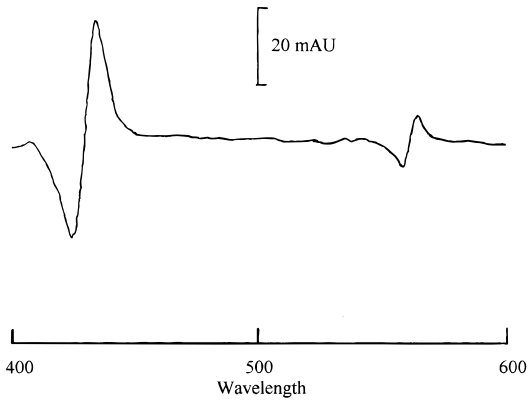

Figure 4. Difference spectrum of **1** binding to reduced cytochrome bc_1. The spectrum represents the binding of 5 μg ml^{-1} **1** to Preparation A of beef heart (fully reduced with dithionite) minus the spectrum of Preparation A of beef heart (fully reduced with dithionite) in the absence of inhibitor. Cytochrome b content was 6.0 μM.

An independent method was used to examine the binding mode of **1** in comparison to inhibitors myxothiazol, antimycin A, rotenone, **2**, and **3**. [^{3}H] **1** was prepared and incubated with rat heart mitochondria in the absence or presence of other additives (Table 4). The results from this direct displacement study indicated that [^{3}H]**1** was strongly displaced by myxothiazol, (*S*+)**1** and **2** (a strobilurin A analog) but was only weakly affected by antimycin A, rotenone, (*R*−) **1** and **3** (an oxazolidinone analog having a large IC_{50} for inhibition of mitochondrial electron transport).

Physiological studies on **1** showed that it was a potent inhibitor of O_2 consumption by zoospores of *P. infestans* (Fig 5). Within a few seconds after the addition of 1 μg ml^{-1} **1** to a zoospore suspension, O_2 consumption was fully inhibited (Fig 5). The IC_{50} for inhibition of O_2 consumption by zoospores was determined as 100 ng ml^{-1}, which was close to its IC_{50} for inhibition of mitochondrial electron transport (45 ng ml^{-1}, Table 3). Microscope observations of *P. infestans* zoospores showed lack of motility within seconds of addition of varying concentrations of **1** to zoospores and, within a minute or two, the

Table 4. Displacement of [^{3}H] **1** from rat heart submitochondria in competitive binding experiments

Addition	*Fraction of specifically bound [^{3}H] **1** retained (±SD)*
None	1.00 (±0.17)
1 (*R*−) (1 μg ml^{-1})	0.88 (±0.080)
2 (*S*+) (1 μg ml^{-1})	0.24 (±0.11)
Myxothiazol (1 μg ml^{-1})	0.41(±0.11)
Antimycin A (1 μg ml^{-1})	0.97 (±0.12)
Antimycin A (10 μg ml^{-1})	1.05(±0.089)
Rotenone (1 μg ml^{-1})	1.01 (±0.047)
Rotenone (10 μg ml^{-1})	1.1[a]
2 (1 μg ml^{-1})	0.29 (±0.15)
3 (1 μg ml^{-1})	1.0(±0.050)

[a] Value from a single measurement.

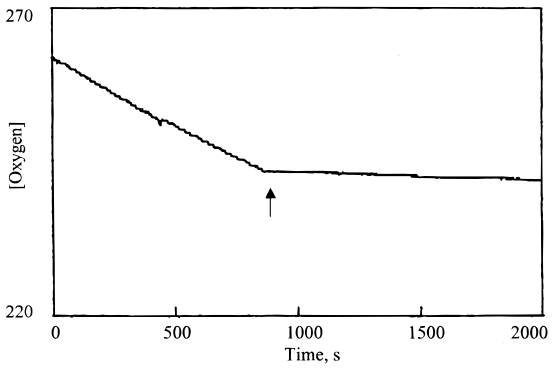

Figure 5. Inhibition by **1** of oxygen consumption in a 2-ml suspension of *Phytophthora infestans* zoospores. Zoospores were incubated at 15°C in an Hansetech oxygen electrode apparatus. After 960 s of measuring the uninhibited rate of O_2 consumption, 2 µl of a 1 mg ml^{-1} ethanolic solution of **1** was added as indicated by the arrow.

zoospores lost membrane integrity and released cellular contents to the surrounding medium (data not shown; see Section 3.2 for famoxadone effects on zoospores).

3.2 Famoxadone mode of action studies

Optimization efforts yielded the more potent fungicide famoxadone which outperformed **1** in greenhouse tests. Many of the biochemical and physiological studies described for **1** above were repeated on famoxadone. Famoxadone was 10-fold more potent than **1** in inhibiting mitochondrial electron transport (NADH to O_2) (Tables 3 and 5) and famoxadone specifically inhibited cytochrome bc_1 rather than complexes I and IV in the NADH to O_2 pathway (Table 2). Famoxadone at 5000 ng ml^{-1} did not inhibit complex II (succinate dehydrogenase). As with **1**, the (*S*−) enantiomer of famoxadone was superior to the (*R*+) enantiomer for inhibition of mitochondrial electron transport (NADH to O_2) in submitochondria, ubiquinol to cytochrome c in purified cytochrome bc_1 from beef heart and for protecting plants from disease (Table 5).

Purified beef heart cytochrome bc_1 reduced with dithionite produced the classical red shift of known inhibitors[5,26] upon treatment with famoxadone (Fig 6 A). Difference spectra of the reduced cytochrome bc_1 more resembled that produced by myxothiazol than that produced by antimycin A: the red shift trough and peaks with both famoxadone or myxothiazol were consistently at 560 and 566 nm, respectively; the red shift trough and peak with antimycin

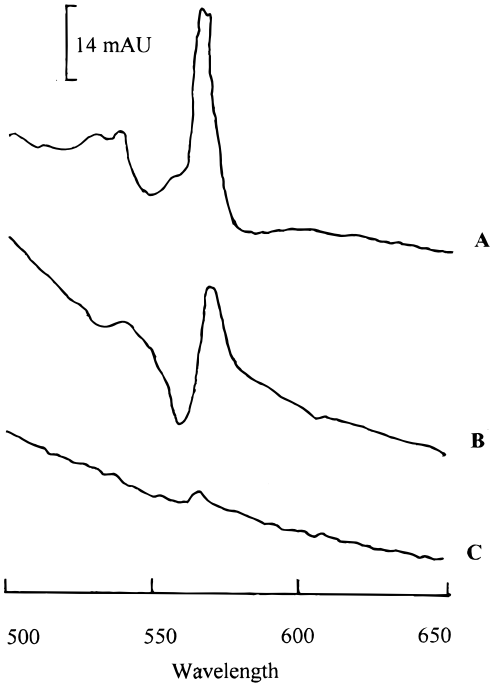

Figure 6. Difference spectra of inhibitors to reduced cytochrome bc_1. The spectra represent the binding of inhibitors to Preparation B of beef heart (6.3 µM with respect to cytochrome b content and fully reduced with dithionite). (A) Spectum of reduced cytochrome bc_1 obtained in the presence of 10 µg ml^{-1} S(−) famoxadone minus the spectrum of reduced cytochrome bc_1 alone. (B) Spectrum of reduced cytochrome bc_1 obtained in the presence of 10 µg ml^{-1} S(−) famoxadone and 10 µg ml^{-1} antimycin A minus the spectrum of reduced cytochrome bc_1 in the presence of 10 µg ml^{-1} antimycin A. (C) Spectrum of reduced cytochrome bc_1 obtained in the presence of 10 µg ml^{-1} S(−) famoxadone and 10 µg ml^{-1} myxothiazol minus the spectrum of reduced cytochrome bc_1 in the presence of 10 µg ml^{-1} S(−) famoxadone.

A were consistently at 558 and 564 nm, respectively. Addition of myxothiazol to the reduced cytochrome bc_1 saturated with 10 µg ml^{-1} (*S*−)famoxadone produced very little change in the difference spectrum in the 500–600 nm region from that before the addition (Fig 6 C) whereas addition of antimycin A to reduced cytochrome bc_1 saturated with 10 µg ml^{-1} (*S*−)famoxadone increased the difference spectrum to such an extent that the spectra of the reduced cytochrome bc_1 saturated with 10 µg ml^{-1} (*S*−)famoxadone and with 10 µg ml^{-1} antimycin A were nearly additive in the spectrum of the reduced cytochrome bc_1 saturated with both inhibitors simultaneously (Fig 6 B). These spectral results strongly suggested that famoxadone occupied an overlapping

Table 5. Selectivity of enantiomers of famoxadone for inhibition of mitochondrial electron transport (NADH to O_2) in rat heart submitochondria, inhibition of cytochrome bc_1 activity (ubiquinol to cytochrome c), and control of grape downy mildew

Compound	*Electron transport* IC_{50} (*ng ml*$^{-1}$) (±*SEM*)	*Cytochrome* bc_1 IC_{50} (*ng ml*$^{-1}$) (±*SEM*)	*Disease control*[a] ED_{90} (*µg ml*$^{-1}$)
Famoxadone (*R*, *S*)	4.5 (±1.4)[b]	28 (±0.6)	3
Famoxadone (*R*+)	200 (±11)	1400 (±140)	10
Famoxadone (*S*−)	2.2 (±0.6)	12 (±0.7)	0.4

[a] ED_{90} values were estimated from plant disease control reports (DuPont Agricultural Products).
[b] Mean and standard deviation from 11 determinations.

binding site with myxothiazol and an entirely independent binding site from antimycin A.

Independent experiments gave further information on the inhibition mode of famoxadone. Figure 7 shows the gx region EPR spectra of the iron-sulfur cluster in ascorbate-reduced cytochrome bc_1 complex in the presence and absence of various inhibitors. Addition of famoxadone to ascorbate-reduced cytochrome bc_1 complex slightly broadens the gx EPR line shape, but the change is quite subtle (Fig 7 A and B). The effect of famoxadone on the gx EPR signal was very similar to that of myxothiazol (Fig 7 C). Von Jagow and Ohnishi[4] have shown that the gx EPR signal of bc_1 complex isolated with Triton X-100 is not very sensitive to myxothiazol, due to the loss of ubiquinol bound to the Q_o site. Our results are consistent with their observation. When stigmatellin was added to the complex, we could clearly see that the gx signal was sharpened and shifted to lower magnetic field. The results suggested that the binding mode of famoxadone to the bc_1 complex is more like that of myxothiazol than that of stigmatellin. We reached the same conclusion when we carried out the EPR experiments using beef heart submitochondria instead of bc_1 complex (data not shown).

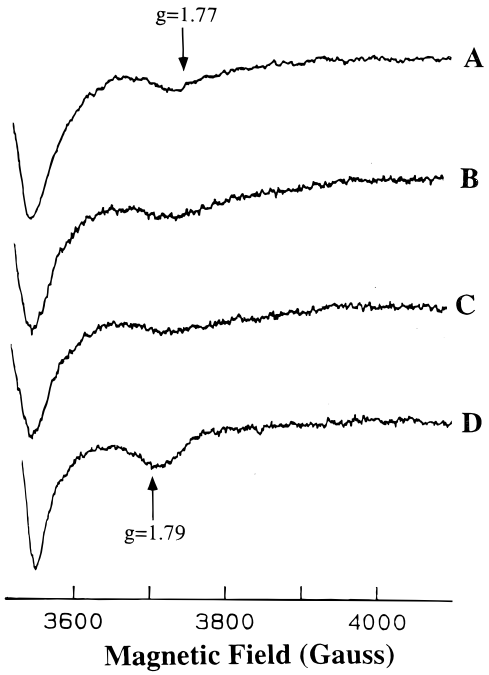

Figure 7. The EPR signal of the gx of the iron-sulfur protein in ascorbate-reduced cytochrome bc_1 complexes treated with various inhibitors: (A) control (no addition of inhibitor); (B) famoxadone; (C) myxothiazol and (D) stigmatellin. The instrument settings were as follows: microwave power, 5 mW; modulation amplitude: 10 G; time constant, 0.128 s; scanning rate, 5G s^{-1}.

Yeast isolates having single amino acid changes in mitochondrial apocytochrome b (obtained from Dr A M Colson)[15] were used for studying their mitochondrial inhibition profiles directly. Whereas the isolates were selected against the inhibitors strobilurin A (mucidin) and myxothiazol, there were no literature reports of their selectivity at the level of mitochondrial electron transport enzyme studies. We isolated mitochondria from the yeast isolates along with their parentals and compared their inhibition properties (Table 6). IC_{50} values for famoxadone, myxathiazol, azoxystrobin and kresoxim-methyl were found to vary differentially within the isolates. Famoxadone inhibition potency was enhanced by the L275F and N256Y alterations more than the other inhibitors which bind to the Q_0 site (azoxystrobin, kresoxim-methyl and myxothiazol). The IC_{50} value for famoxadone was decreased by 19-fold by the L275F change, whereas the IC_{50} for azoxystrobin was decreased by only 1.6-fold and the IC_{50} values for kresoxim-methyl and myxothiazol increased. The N256Y change increased the potency of famoxadone by 2-fold, while azoxystrobin's potency was increased by 1-fold and the potencies of kresoxim-methyl and myxothiazol were only slightly enhanced. Three of the amino acid changes studied (G137R,

Table 6. IC_{50} values and IC_{50} values (relative to the parental values) for inhibition of mitochondrial electron transport in yeast mitochondria having single amino acid changes in cytochrome b

	IC_{50} (ng ml^{-1})[a] (±SEM)				
Yeast isolate	*Famoxadone*	*Azoxystrobin*	*Kresoxim-methyl*	*Myxothiazol*	*Antimycin A*
D225-5A[b]	32 (±1.3)	4.0 (±0.32)	1.7 (±0.03)	0.98 (±0.06)	2.0 (±0.30)
(Parental)	(1)	(1)	(1)	(1)	(1)
MUC1-771	120 (±4.7)	270 (±12)	310 (±52)	21 (±1.3)	3.7 (±0.20)
(G137R)	(3.8)	(68)	(180)	(21)	(1.8)
MUC2-772	71 (±4.4)	6.1 (±0.99)	44 (±2.4)	14 (±0.8)	1.8 (±0.10)
(L275S)	(2.2)	(1.5)	(24)	(14)	(0.9)
GM50-3C[c]	45 (±0.91)	17 (±0.38)	1.3 (±0.06)	2.0 (±0.08)	3.6 (±0.40)
(Parental)	(1)	(1)	(1)	(1)	(1)
MYX1-103	590 (±24)	37 (±3.8)	12 (±0.56)	130 (±6.4)	4.3 (±1.1)
(F129L)	(13)	(2.2)	(9.2)	(64)	(1.2)
MYX-2	2.2 (±0.26)	6.5 (±0.41)	2.3 (±0.27)	2.6 (±0.20)	2.8 (±0.10)
(L275F)	(0.05)	(0.38)	(1.8)	(1.3)	(0.78)
MYX3-119	14 (±0.57)	8.3 (±0.51)	1.2 (±0.07)	1.5 (±0.20)	2.8??? (±0.10)
(N256Y)	(0.31)	(0.49)	(0.92)	(0.75)	(0.78)

[a] IC_{50} relative to parental value in parentheses.
[b] Parental for MUC1-771 and MUC2-772.
[c] Parental for MYX1-103, MYX2-124 and MYX3-119.

Table 7. Dependence of IC_{50} for inhibition of mitochondrial electron transport (NADH to O_2) on the biological source of submitochondria

Mitochondrial source	*IC_{50} ($ng\,ml^{-1}$) ($\pm SEM$)*		
	Famoxadone	*Myxothiazol*	*Antimycin A*
Rat Heart	4.5 ($\pm$1.4)[a]	1.2 ($\pm$0.1)	1.4 ($\pm$0.2)
Beef Heart	6.2 ($\pm$0.9)	1.5 ($\pm$0.07)	1.6 ($\pm$0.1)
P. infestans	2.8 ($\pm$0.6)	ND[b]	ND[b]
S. cerevisiae	46 ($\pm$4)	1.0 ($\pm$0.1)	1.4 ($\pm$0.06)
Cauliflower	53 ($\pm$9)	13 ($\pm$0.5)	3.0 ($\pm$0.4)
Neurospora crassa	19 ($\pm$0.05)	4.0 ($\pm$0.4)	6.0 ($\pm$0.8)
Achlya sp.	7.1 ($\pm$0.7)	2.7 ($\pm$0.2)	5.8 ($\pm$0.1)
Magnaporthe grisea	9.0 ($\pm$0.3)	ND[b]	ND[b]

[a] Mean and standard deviation from 11 determinations.
[b] ND, not determined.

L275S and F129L) decreased the potency of all Q_0 site inhibitors differentially. Notably, G137R decreased potencies of azoxystrobin, kresoxim-methyl and myxothiazol by 67-, 179- and 20-fold, respectively, and that of famoxadone by only 2.8-fold. It should be noted that IC_{50} values for antimycin A, which binds to the Q_i site, should be equal for the parentals and the isolates, which have single amino acid changes affecting the Q_0 site. Deviations of IC_{50} values for antimycin A (relative to the parental values) from 1.0 should be considered as errors in estimating and comparing IC_{50} values.

Famoxadone was found to be a general poison of mitochondrial electron transport among many species (Table 7), as were myxothiazol and antimycin A. Fungal growth studies showed famoxadone to be a general fungicide (Table 8) even though the compound was more useful for controlling specific diseases in greenhouse studies.

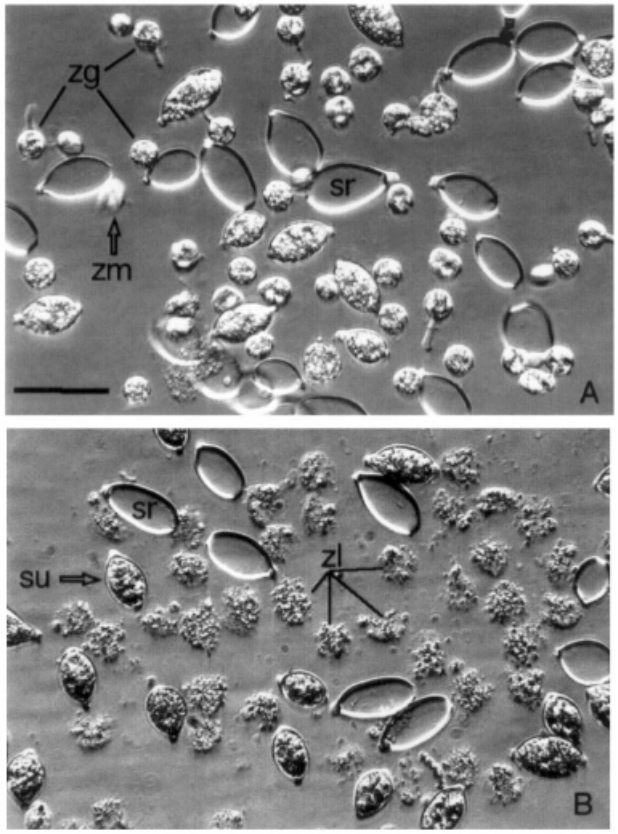

Figure 8. Disruption of *Phytophthora infestans* zoospores following famoxadone incubations. (A) Sporangia and zoospores in the absence of famoxadone. (B) Sporangia and zoospores in the presence of famoxadone. Scale bar = 10 μm.
sr = sporangium, zoospores released; su = sporangium, undifferentiated; zg = zoospore, lysed; zm = zoospore, motile.

Table 8. Inhibition of fungal growth by famoxadone and azoxystrobin

Organism	*IC_{50} ($ng\,ml^{-1}$) ($\pm SEM$)*	
	Famoxadone	*Azoxystrobin*
Rhizoctonia solani	170 ($\pm$70)	14 ($\pm$7)
Magnaporthe grisea	110 ($\pm$20)	38 ($\pm$17)
Septoria nodorum	6 ($\pm$1)	15 ($\pm$2)
Phytophthora infestans	$<$1.5	21 ($\pm$8)
Neurospora crassa	12 ($\pm$1)	25 ($\pm$6)

Growth studies of *S. cerevisiae* on different carbon sources were conducted to examine further the mode of action of famoxadone at the physiological level. When the yeast was grown in media containing non-fermentable carbon sources, glycerol and ethanol, famoxadone was a potent inhibitor of growth, having IC_{50} values below 1 $\mu g\,ml^{-1}$ (Table 9). When the yeast was grown on a fermentable carbon source, glucose, famoxadone was a much weaker inhibitor of

Table 9. Dependence of inhibition of growth of Saccharomyces cerevisiae on carbon source

Compound	*IC_{50} ($\mu g\,ml^{-1}$) ($\pm SEM$)*		
	Carbon source		
	Glucose	*Ethanol*	*Glycerol*
Famoxadone	12.0 ($\pm$1.5)	0.36 ($\pm$0.40)	0.18 ($\pm$0.10)
Azoxystrobin	4.4 ($\pm$0.6)	$<$0.1	$<$0.1
Kresoxim-methyl	4.4 ($\pm$0.6)	$<$0.1	$<$0.1
Metominostrobin	7.5 ($\pm$1.0)	0.21 ($\pm$0.20)	0.18 ($\pm$0.10)
Antimycin A	5.8 ($\pm$0.7)	$<$0.1	$<$0.1

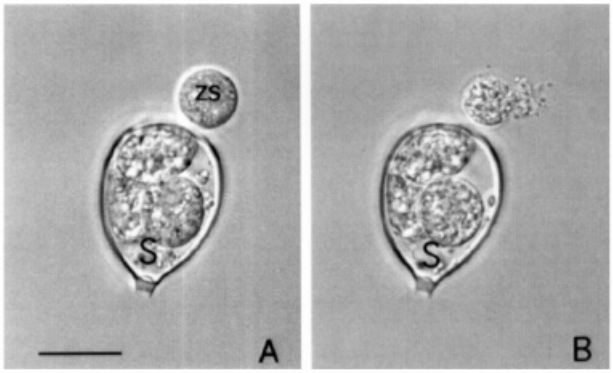

Figure 9. Disruption of *Plasmopara viticola* zoospores by famoxadone incubations. (A) Sporangium and zoospore prior to lysis. (B) Sporangium and zoospore immediately after lysis. Scale bar = 50 μm. s = sporangium; zs = zoospore.

growth, having an IC_{50} value of 12 μg ml^{-1}. Similar trends were found for other cytochrome bc_1 inhibitors antimycin A, azoxystrobin, kresoxim-methyl and **4**. The results from this classical experimental protocol fully support the mode of action studies described above for famoxadone as a poison of mitochondrial electron transport.

Famoxadone is being marketed for controlling late blight disease and grape downy mildew, among other diseases. We examined the effects of famoxadone on zoospores from the disease determinants of late blight (*Phytophthora infestans*) and grape downy mildew (*Plasmopara viticola*). As was found with **1**, addition of famoxadone to suspensions of *Phytophthora infestans* zoospores immediately curtailed O_2 consumption by the single-celled zoospore and the IC_{50} for inhibition was determined as 100 ng ml^{-1}.

Microscope studies on *Phytophthora infestans* and *Plasmopara viticola* indicated that the freely swimming zoospores lose motility within seconds of adding famoxadone to suspensions of zoospores. Often the zoospores were observed to release their scissor-like flagella after motility was lost upon treatment with famoxadone. Within minutes of treatments the zoospores were observed to lose membrane integrity and release cellular contents to the surrounding medium (Figs 8 and 9).

4 DISCUSSION

In Section 3 we believe compelling evidence has been provided for the assignment of cytochrome bc_1 as the biochemical target of famoxadone. Famoxadone was a potent inhibitor of mitochondrial electron transport (NADH to O_2), specifically inhibiting the catalytic function of cytochrome bc_1. Reduced difference spectra of cytochrome bc_1 strongly suggested that famoxadone bound in the Q_o pocket of cytochrome bc_1, overlapping the binding site of myxothiazol. Single amino acid changes in yeast mitochondrial apocytochrome b affecting the binding of Q_o inhibitors also affected the IC_{50} values of famoxadone. EPR experiments suggested that the binding mode of famoxadone was more like that of myxothiazol than that of stigmatellin. Results from physiological experiments showed that famoxadone inhibited O_2 consumption by zoospores of the target organism *P. infestans*, it inhibited growth of the yeast, *S. cerevisiae*, much more strongly when the medium contained a nonfermentable carbon source than in media containing a fermentable carbon source and zoospores lost motility and were destroyed when famoxadone inhibited their energy metabolism.

The early studies on **1** remain useful to our understanding of famoxadone function. **1** was studied thoroughly enough to conclude that it had very similar properties to famoxadone and some of the experiments on **1** may be extended to famoxadone. For example, the incorporation of amino acids, ribonucleosides and deoxyribonucleosides into their respective polymers was strongly inhibited by **1**. Even though a similar study was not conducted on famoxadone, we may infer that a similar result would have been obtained. The IC_{50} for inhibition of mitochondrial electron transport (NADH to O_2) in mitochondria isolated from *P. infestans* was 6-fold larger for **1** than that for famoxadone, yet the IC_{50} values for inhibition of O_2 consumption in zoospores of *P. infestans* were nearly identical. We attribute the discrepancy to a kinetic effect in that the larger molecule, famoxadone, may be slower in reaching its biochemical target within the cells of zoospores than **1**. Furthermore, the overlapping studies on famoxadone and **1** allow us to have reasonable confidence interpretating closer analogs of famoxadone (in comparison to **1**) as having the same biochemical mode of action and differences in biological performances may be interpreted accordingly.

It was not an overwhelming surprise to find that famoxadone exhibited a different binding mode to mitochondrial cytochrome b from other cytochrome b ligands known to bind near the Q_o domain of the protein, as shown in Table 6. Of the five amino acid changes in yeast apocytochrome b that were studied, three decreased the binding potency of famoxadone and two enhanced it. In the three examples where famoxadone binding potency was decreased by amino acid changes (F129L, G137R and L275S), the potency of famoxadone was reduced by an average of 6.3-fold, whereas the potencies of azoxystrobin, kresoxim-methyl and myxothiazol were reduced by an average, 24-, 71- and 33-fold respectively. Comparison of the extremes in altered binding constants is informative: G137R decreased famoxadone binding potency by 2.8-fold but decreased that of kresoxim-methyl by 180-fold; L275S decreased famoxadone binding potency by 1.2-fold but decreased that of kresoxim-methyl by 23-fold; F129L decreased famoxadone binding potency by 12-fold but decreased that of myxothiazol by 63-fold; L275 increased famoxadone binding potency

by 19-fold but decreased that of kresoxim-methyl by 0.8-fold; and N256Y increased famoxadone binding potency by 2.2-fold but increased that of kresoxim-methyl by only 0.1-fold. Many species have apocytochrome b sequences which correspond to the yeast numbering G137, F275, F129 and N256. Two notable exceptions are *S. cerevisiae* and *N. crassa*, both of which have L275 instead of F275 and this difference probably accounts for the 5- to 10-fold decrease in sensitivity to famoxadone in submitochondria isolated from these species in comparison to submitochondria from other species (Table 7).

The molecular structures of famoxadone, azoxystrobin, kresoxim-methyl and myxothiazol alone suggest that their specific binding modes to cytochrome b of cytochrome bc_1 should differ. It is hoped that, in the near future, sufficiently high resolution X-ray structures of these Q_o ligands bound to cytochrome bc_1 may be obtained so that physical interpretations of our results may be elucidated.

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